

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

The Journal of Clinical Investigation

January 1, 1996, Volume 97, Number 1

Exhibit 13

Editorials

The times they are still a'changing: keeping up with the times.

A.P. Varki 1

Molecular Medicine in genetically engineered animals: series introduction.

K.R. Chien 2

Perspectives

Gene modification via "plug and socket" gene targeting. J. Lewis, B. Yang,

P. Deloff, and O. Smithies 3

Regular Articles

Digoxin reduces β -adrenergic contractile response in rabbit hearts.

Ca^{2+} -dependent inhibition of adenylyl cyclase activity via $\text{Na}^+/\text{Ca}^{2+}$ exchange. K. Nagai, T. Murakami, T. Iwase, T. Tomita, and S. Sasayama 6

Sex steroids, bone mass, and bone loss. A prospective study of pre-,

peri-, and postmenopausal women. C. Slemenda, C. Longcope, M. Peacock, S. Hui, and C.C. Johnston 14

Vitamin C improves endothelium-dependent vasodilation in patients

with non-insulin-dependent diabetes mellitus. H.H. Ting, F.K. Timimi, K.S. Boles, S.J. Creager, P. Ganz, and M.A. Creager 22

Red cell membrane remodeling in sickle cell anemia. Sequestration of

membrane lipids and proteins in heinz bodies. S.-C. Liu, S.J. Yi, J.R. Mehta, P.E. Nichols, S.K. Ballas, P.W. Yacono, D.E. Golan, and J. Palek 29

Distribution and regulation of plasminogen activator inhibitor-1 in

murine adipose tissue in vivo. Induction by tumor necrosis factor- α and lipopolysaccharide. F. Samad, K. Yamamoto, and D.J. Loskutoff 37

Functional magnetic resonance imaging reveals brain regions mediating

the response to resistive expiratory loads in humans. D. Gozal, O. Onidvar, K.A.T. Kirlew, G.M. Hathout, R.B. Lufkin, and R.M. Harper 47

Modulation of L-Selectin ligand expression during an immune response

accompanying tumorigenesis in transgenic mice. S.V. Onrust, P.M. Hariri, S.D. Rosen, and D. Hanahan 54

Prolonged postprandial responses of lipids and apolipoproteins in

triglyceride-rich lipoproteins of individuals expressing an apolipoprotein $\epsilon 4$ allele. N. Bergeron and R.J. Havel 65

Accumulation of methotrexate polyglutamates in lymphoblasts is a

determinant of antileukemic effects in vivo. A rationale for high-dose methotrexate. E. Masson, M.V. Relling, T.W. Synold, Q. Liu, J.D. Schuetz, J.T. Sandlund, C.-H. Pui, and W.E. Evans 73

Comparison of the time courses of insulin and the portal signal on

hepatic glucose and glycogen metabolism in the conscious dog. M.J. Pagliassotti, L.C. Holste, M.C. Moore, D.W. Neal, and A.D. Cherrington 81

Gastric emptying and release of incretin hormones after glucose

ingestion in humans. J. Schirra, M. Katschinski, C. Weidmann, T. Schäfer, U. Wank, R. Arnold, and B. Göke 92

Increased δ aminolevulinic acid and decreased pineal melatonin

production H. Puy, J.-C. Deybach, A. Bogdan, J. Callebaut, M. Baumgartner, P. Voisin, Y. Nordmann, and Y. Tavitou 104

IgG antiendothelial cell autoantibodies from scleroderma patients

induce leukocyte adhesion to human vascular endothelial cells in vitro. D. Carvalho, C.O.S. Savage, C.M. Black, and J.D. Pearson 111

Role of angiotensin II in dietary modulation of rat late distal tubule

bicarbonate flux in vivo. D.Z. Levine, M. Iacovitti, S. Buckman, and K.D. Burns 120

Direct assessment of liver glycogen storage by ^{13}C nuclear magnetic

resonance spectroscopy and regulation of glucose homeostasis after a mixed meal in normal subjects. R. Taylor, I. Magnusson, D.L. Rothman, G.W. Cline, A. Caiano, C. Cobelli, and G.I. Shulman 126

Elimination of the action of glucagon-like peptide 1 causes an impairment of glucose tolerance after nutrient ingestion by healthy baboons. D.A. D'Alessio, R. Vogel, R. Prigeon, E. Laschansky, D. Koerker, J. Eng, and J.W. Ensinck 133

G-protein coupled and tyrosine kinase receptors: evidence that activation of the insulin-like growth factor I receptor is required for thrombin-induced mitogenesis of rat aortic smooth muscle cells.

P. Delafontaine, A. Anwar, H. Lou, and L. Ku 139

Induction of cyclin gene expression by homocysteine in vascular

smooth muscle cells. J.-C. Tsai, H. Wang, M.A. Perrella, M. Yoshizumi, N.E.S. Sibinga, L.C. Tan, E. Haber, T.H.-T. Chang, R. Schlegel, and M.-E. Lee 146

Profound induction of hepatic cholesteryl ester transfer protein

transgene expression in apolipoprotein e and low density lipoprotein receptor gene knockout mice. L. Masucci-Magoulas, A. Plump, X.C. Jiang, A. Walsh, J.L. Breslow, and A.R. Tall 154

Involvement of wound-associated factors in rat brain astrocyte

migratory response to axonal injury: in vitro simulation. A. Faber-Elman, A. Solomon, J.A. Abraham, M. Marikovsky, and M. Schwartz 162

Inhibition of cyclic 3'-5'-guanosine monophosphate-specific

phosphodiesterase selectively vasodilates the pulmonary circulation in chronically hypoxic rats. A.H. Cohen, K. Hanson, K. Morris, B. Fouhy, I.F. McMurtry, W. Clarke, and D.M. Rodman 172

Mechanism of impaired glucose-potentiated insulin secretion in

diabetic 90% pancreatectomy rats. Study using glucagonlike peptide-1 (7-37). Y.A. Hosokawa, H. Hosokawa, C. Chen, and J.L. Leahy 180

Heparin-binding secretory transforming gene (*hst*) facilitates rat

lactotrope cell tumorigenesis and induces prolactin gene transcription. I. Shimon, A. Hütner, J. Said, O.M. Spirina, and S. Melmed 187

A single strand conformation polymorphism study of cd40 ligand.

Q. Liu, J. Rohrer, R.C. Allen, M. Larché, J.M. Greene, A.O. Shigeoka, R.A. Gatti, D.C. Derauf, J.W. Belmont, and M.E. Conley 196

Maternal vasoactive intestinal peptide and the regulation of embryonic

growth in the rodent. J.M. Hill, S.K. McCune, R.J. Alvero, G.W. Glazner, K.A. Henius, S.F. Stanziale, J.R. Keimowitz, and D.E. Brememan 202

Cerebral protection in homozygous null ICAM-1 mice after middle

cerebral artery occlusion. Role of neutrophil adhesion in the pathogenesis of stroke. E.S. Connolly, Jr., C.J. Winfree, T.A. Springer, Y. Naka, H. Liao, S.D. Yan, D.M. Stern, R.A. Solomon, J.-C. Gutierrez-Ramos, and D.J. Pinsky 209

Allogeneic hematolymphoid microchimerism and prevention of

autoimmune disease in the rat. A relationship between allo- and autoimmunity. C.P. Delaney, N. Murase, M. Chen-Woan, J.J. Fung, T.E. Starzl, and A.J. Demetris 217

T.E. Starzl and A.J. Demetris 217

T.E. Starzl and A.J. Demetris 217

T.E. Starzl and A.J. Demetris 217

T.E. Starzl and A.J. Demetris 217

T.E. Starzl and A.J. Demetris 217

T.E. Starzl and A.J. Demetris 217

T.E. Starzl and A.J. Demetris 217

T.E. Starzl and A.J. Demetris 217

T.E. Starzl and A.J. Demetris 217

T.E. Starzl and A.J. Demetris 217

T.E. Starzl and A.J. Demetris 217

T.E. Starzl and A.J. Demetris 217

T.E. Starzl and A.J. Demetris 217

T.E. Starzl and A.J. Demetris 217

T.E. Starzl and A.J. Demetris 217

T.E. Starzl and A.J. Demetris 217

T.E. Starzl and A.J. Demetris 217

T.E. Starzl and A.J. Demetris 217

T.E. Starzl and A.J. Demetris 217

T.E. Starzl and A.J. Demetris 217

T.E. Starzl and A.J. Demetris 217

T.E. Starzl and A.J. Demetris 217

T.E. Starzl and A.J. Demetris 217

T.E. Starzl and A.J. Demetris 217

T.E. Starzl and A.J. Demetris 217

T.E. Starzl and A.J. Demetris 217

T.E. Starzl and A.J. Demetris 217

The potent anti- <i>staphylococcus aureus</i> activity of a sterile rabbit inflammatory fluid is due to a 14-kD phospholipase A2. <i>Y. Weinrauch, P. Elsbach, L. M. Madsen, A. Foreman, and J. Weiss</i>	250
Human diabetes associated with defects in nuclear regulatory proteins for the insulin receptor gene. <i>A. Brunetti, L. Brunetti, D. Foti, D. Accili, and I. D. Goldfine</i>	258

Na ⁺ /Myo-inositol transport is regulated by basolateral tonicity in madin-darby canine kidney cells. <i>A. Yamauchi, T. Sugiura, T. Ito, A. Miyai, M. Horio, E. Imai, and T. Kamada</i>	263
<i>Author Index</i>	269
<i>Correction</i>	271

Bleomycin-induced Pulmonary Fibrosis in Transgenic Mice That either Lack or Overexpress the Murine Plasminogen Activator Inhibitor-1 Gene

Daniel T. Eitzman,* Ronald D. McCoy,† Xianxian Zheng,§ William P. Fay,* Tingliang Shen,§ David Ginsburg,§
and Richard H. Simon*

*Divisions of Cardiology, †Pulmonary and Critical Care Medicine, and §Human Genetics, Department of Internal Medicine, and the Howard Hughes Medical Institute, University of Michigan Medical Center, Ann Arbor, Michigan 48109

Abstract

Impaired fibrinolytic activity within the lung is a common manifestation of acute and chronic inflammatory lung diseases. Because the fibrinolytic system is active during repair processes that restore injured tissues to normal, reduced fibrinolytic activity may contribute to the subsequent development of pulmonary fibrosis. To examine the relationship between the fibrinolytic system and pulmonary fibrosis, lung inflammation was induced by bleomycin in transgenic mice that either overexpressed or were completely deficient in murine plasminogen activator inhibitor-1 (PAI-1). 2 wk after 0.075 U of bleomycin, the lungs of transgenic mice overexpressing PAI-1 contained significantly more hydroxyproline ($118 \pm 8 \mu\text{g}$) than littermate controls ($70.5 \pm 8 \mu\text{g}$, $P < 0.005$). 3 wk after administration of a higher dose of bleomycin (0.15 U), the lung hydroxyproline content of mice completely deficient in PAI-1 ($49 \pm 8 \mu\text{g}$) was not significantly different ($P = 0.63$) than that of control animals receiving saline ($37 \pm 1 \mu\text{g}$), while hydroxyproline content was significantly increased in heterozygote ($77 \pm 12 \mu\text{g}$, $P = 0.06$) and wild-type ($124 \pm 19 \mu\text{g}$, $P < 0.001$) littermates. These data demonstrate a direct correlation between the genetically determined level of PAI-1 expression and the extent of collagen accumulation that follows inflammatory lung injury. These results strongly support the hypothesis that alterations in fibrinolytic activity influence the extent of pulmonary fibrosis that occurs after inflammatory injury. (*J. Clin. Invest.* 1996; 97:232–237.) Key words: fibrinolysis • fibrin • plasmin • interstitial lung disease • collagen

Introduction

Successful repair of damaged alveoli during inflammatory lung diseases requires replacement of injured alveolar cells, restora-

tion of damaged extracellular matrix, and clearance of plasma proteins that have leaked into the alveolar space. Of critical importance to these repair processes is plasmin, a broad spectrum protease which is involved in cell migration, modulation of inflammatory activity, and breakdown of fibrin and other extracellular proteins. This latter function of plasmin may be important for limiting scar formation by dismantling the provisional matrix on which fibroblasts invade and secrete interstitial collagens. The normal alveolar space has net fibrinolytic activity due to the presence of urokinase-type plasminogen activator (u-PA)¹ (1, 2). However, during many acute and chronic inflammatory lung disorders, fibrin accumulates in lung tissue (3–9). The fibrinolytic activity in bronchoalveolar lavage (BAL) fluid from patients with the adult respiratory distress syndrome (1, 2), idiopathic pulmonary fibrosis (10), sarcoidosis (10, 11), and bronchopulmonary dysplasia (12) has been found to be suppressed. All of the above diseases have been associated with the development of pulmonary fibrosis. A similar pattern of depressed fibrinolysis can be seen in a variety of animal models of lung injury (13–15).

Plasminogen activator inhibitor-1 (PAI-1) is the major inhibitor of plasminogen activators in plasma (16) and the alveolar space (2). PAI-1, a member of the serine protease inhibitor (serpin) gene family, rapidly inhibits both u-PA and tissue-type plasminogen activator (t-PA) by forming 1:1 protease-inhibitor complexes that are enzymatically inactive (16). Complete deficiency of PAI-1 in a human was associated with abnormal bleeding (17), and mice that are deficient in PAI-1 display enhanced fibrinolytic activity (18). Conversely, increased plasma PAI-1 levels in humans are associated with thrombotic events (19, 20). Elevated levels of PAI-1 have been observed in BAL specimens obtained from patients with adult respiratory distress syndrome and have been shown to reduce the fibrinolytic capacity of the fluid (1, 2).

Although previous experiments have demonstrated an association of increased PAI-1 levels and pulmonary fibrosis, the primary role of PAI-1 in this process has not been established. To determine whether alterations in systemic PAI-1 levels affect the development of pulmonary fibrosis, lung injury was induced by bleomycin in transgenic mice that were engineered to either overexpress or be completely deficient in PAI-1. The level of PAI-1 gene expression was observed to strongly correlate with the amount of collagen accumulation within lung tissues, suggesting that the balance of fibrinolytic activity within

Address correspondence to Richard H. Simon, M.D., 3110B Taubman Center, Box 1698, University of Michigan Medical Center, Ann Arbor, MI 48109-1698. Phone: 313-936-4570; FAX: 313-936-7024; E-mail: rsimon@umichmed.umich.edu

Received for publication 30 March 1995 and accepted in revised form 17 October 1995

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.
0021-9758/96/010232-06 \$2.00

Volume 97, Number 1, January 1996, 232–237

1. Abbreviations used in this paper: BAL, bronchoalveolar lavage; PAI-1, plasminogen activator inhibitor-1; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator.

the lung is an important determinant of the pulmonary response to inflammatory injury.

Methods

Animals. All animal experiments were performed in accordance with institutional guidelines set forth by the University committee on the use and care of animals. PAI-1-deficient mice generated by homologous recombination were a generous gift from D. Collen and P. Carmeliet (University of Leuven, Belgium) (21). PAI-1-deficient (-/-) mice, along with PAI-1 heterozygote (+/-) and PAI-1 wild-type (+/+) littermates, were generated by a cross between heterozygote animals. Genotyping was performed by PCR analysis of tail DNA specimens obtained at 3 wk of age (22). Primers which annealed to bp 1007-1031 (5') and 1503-1532 (3') of the neomycin resistance gene were used to recognize the knock-out construct, while primers designed to anneal to bp 1263-1286 (5') and 1473-1496 (3') (across intron H) of the PAI-1 cDNA sequence were used to recognize the native gene. Thus, DNA samples from mice heterozygous for PAI-1 displayed two different PCR products (496 and 520 bp).

Transgenic mice overexpressing a murine PAI-1 minigene under the direction of the CMV promoter were generated previously (Shen, T., and D. Ginsburg, manuscript in preparation). Serum levels of PAI-1 in mice carrying the PAI-1 transgene were 264 ± 53 vs. 11 ± 4 ng/ml in littermate controls. PAI-1 levels in lung homogenates of transgenic mice were 1.448 ± 320 ng/gram wet tissue compared with 376 ± 38 ng/gram in littermate controls. The PAI-1 transgenic and normal littermate control animals that were used in the experiments were the products of crosses between the transgenic founder animal (SJL/J) and a C57BL/6 mate. The presence of the PAI-1 transgene in individual mice was determined using PCR with the PAI-1 primers as described above. Two products of different sizes were generated in the presence of transgene: a 496-bp product from the normal allele, and a 233-bp product from the transgene, which lacks an intervening intron. Only the 496-bp product was observed in animals lacking the transgene.

Bleomycin treatment. Male and female 4-6-wk-old mice, weighing an average of 20 ± 3 grams, were maintained in a specific pathogen-free environment and fed ad libitum. Intratracheal administration of bleomycin to mice under intraperitoneal ketamine anesthesia was performed with the operator blinded to genetic status of mice, as described previously (23). After tracheostomy, bleomycin (Bristol Laboratories, Syracuse, NY) was instilled at doses of 0.075 or 0.15 U in a final volume of 50 μ l. These doses were determined from preliminary experiments with mice of similar genetic background to consistently produce pulmonary fibrosis with a low mortality rate ($< 25\%$).

Hydroxyproline assay. To estimate total lung collagen content, hydroxyproline was measured as previously described with modifications (24). The lung vasculature was perfused free of blood by slowly injecting 3 ml of PBS into the right ventricle. The left lung was then excised and homogenized (Tissue Tearor; Biospec Products, Inc., Bartlesville, OK) in 2 ml of PBS. A 1-ml aliquot was desiccated using rotary vacuum pump (Savant Instruments, Inc., Farmingdale, NY) and then hydrolyzed in 6 N HCl at 110°C for 12 h. 50- μ l aliquots were added to 1 ml of 1.4% chloramine T (Sigma Chemical Co., St. Louis, MO), 10% *n*-propanol, and 0.5 M sodium acetate, pH 6.0. After 20 min of incubation at room temperature, 1 ml of Erlich's solution (1 M *p*-dimethylaminobenzaldehyde in 70% *n*-propanol, 20% perchloric acid) was added and allowed to incubate at 65°C for 15 min. Absorbance was measured at 550 nm and the amount of hydroxyproline was determined against a standard curve generated using known concentrations of reagent hydroxyproline (Sigma Chemical Co.). The ability of the assay to completely hydrolyze and recover hydroxyproline from collagen was confirmed using samples containing known amounts of purified collagen (Nitrogen-100; Caltrex Laboratories, Palo Alto, CA).

Histology and immunohistochemistry. After the left lung of each

animal was removed for hydroxyproline assay, 0.5 ml 4% paraformaldehyde was instilled intrabronchially into the right lung, the airway was ligated, and the lung was submerged in the same fixative. The tissue was embedded in paraffin, and histologic sections were viewed after Masson trichrome staining. Immunostaining of fibrin was performed on paraffin-embedded sections that had been fixed in 4% paraformaldehyde. Goat anti-mouse serum was purchased from Nordic Immunological Laboratories (Capistrano Beach, CA). The IgG fraction was purified by passage over a protein A-Sepharose column (Pharmacia, Uppsala, Sweden) and used at a concentration of 10 μ g/ml. Binding of primary antibody was detected using a biotinylated rabbit anti-goat antibody and a Histostain-SP Kit (Zymed Laboratories, South San Francisco, CA). To evaluate the specificity of the immunostaining, preimmune goat IgG (10 μ g/ml) was used as a control condition.

Statistics. Values are expressed as mean \pm SEM. Differences between genetically altered mice and littermate controls were analyzed using ANOVA with Fisher's PLSD test for pair-wise comparisons (Statview 4.0; Abacus Concepts, Inc., Berkeley, CA). A *P* value < 0.05 was considered statistically significant.

Results

Collagen content of bleomycin-treated lungs. 2 wk after intratracheal bleomycin (0.075 U), lung collagen, as measured by hydroxyproline content, increased in both PAI-1 overexpressing mice ($P < 0.001$) and their normal littermate controls ($P < 0.03$) (Fig. 1). However, the increase in collagen induced by

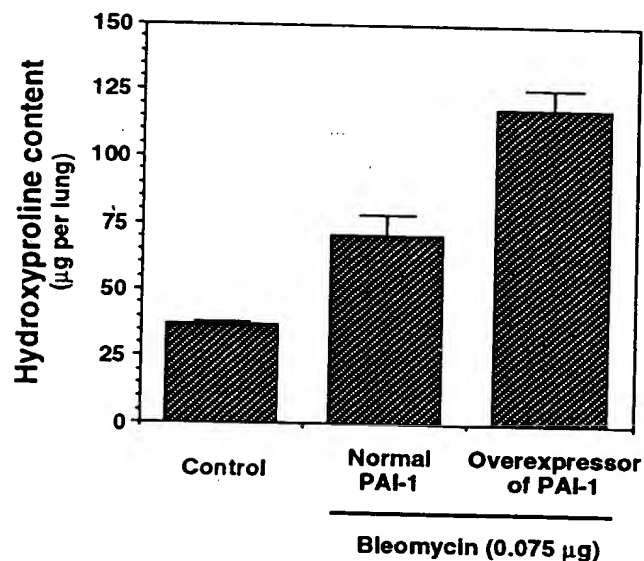


Figure 1. Effect of intratracheal bleomycin on lung hydroxyproline content in PAI-1-overexpressing mice and normal littermates. Bleomycin (0.075 U) or PBS was instilled intratracheally into normal or transgene PAI-1-overexpressing mice, and 2 wk later lung hydroxyproline content was measured as outlined in Methods. The bleomycin-induced increase in lung hydroxyproline of transgenic mice overexpressing PAI-1 ($n = 3$) was greater ($P < 0.002$) than that seen in the bleomycin-treated normal PAI-1 littermates ($n = 3$). The hydroxyproline content of both bleomycin-treated groups was greater ($P < 0.005$) than that of PBS-treated control mice lacking the overexpressing murine PAI-1 transgene ($n = 2$). Data from other experiments revealed that lung hydroxyproline content did not differ between normal and PAI-1-overexpressing mice treated with PBS.

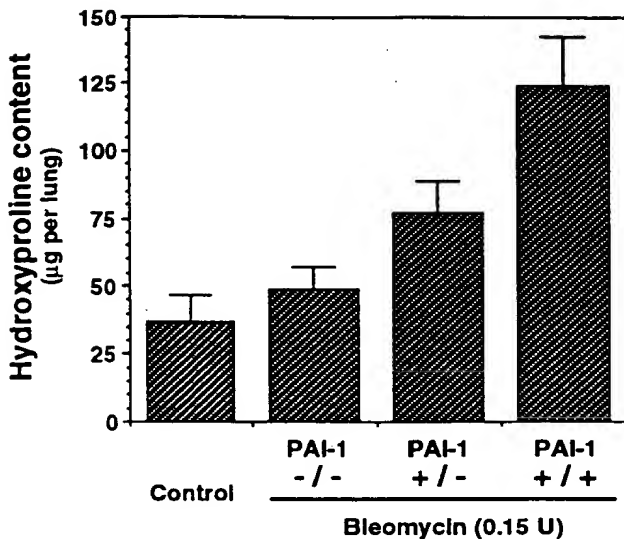


Figure 2. Effect of intratracheal bleomycin on lung hydroxyproline content in PAI-1-deficient mice and littermates. Bleomycin (0.15 U) or PBS was instilled intratracheally into normal (PAI-1 +/+), heterozygous PAI-1-deficient (PAI-1 +/-), or homozygous PAI-1-deficient (PAI-1 -/-) mice. 3 wk later, lung hydroxyproline content was measured as outlined in Methods. The lung hydroxyproline content of bleomycin-treated mice was directly related to PAI-1 gene dose ($r = 0.72$, $P < 0.001$) with the level in normal (PAI-1 +/+) mice being significantly greater than PAI-1 +/- ($n = 6$, $P < 0.02$) and PAI-1 -/- ($n = 6$, $P < 0.001$) mice. The hydroxyproline content of bleomycin-treated PAI-1 -/- mice was not significantly different from that of PBS-treated PAI-1 +/- littermates (Control, $n = 4$, $P < 0.63$).

bleomycin in mice carrying the PAI-1 transgene (3.2 times PBS control animals) was significantly greater ($P < 0.005$) than the increase seen in the transgene-negative littermates (1.9 times PBS controls). No significant differences in hydroxyproline content were observed between normal and transgene-positive mice when no treatment or only PBS was administered (data not shown).

Mice deficient in PAI-1 were next studied to determine if they would develop less collagen in response to bleomycin. For these experiments a higher dose of bleomycin (0.15 U) was used to more clearly discern a protective effect of PAI-1 deficiency. 3 wk after wild-type (PAI-1 +/+) mice were given bleomycin, lung hydroxyproline content increased significantly compared with mice that received only PBS ($P < 0.001$) (Fig. 2). In contrast, PAI-1 -/- mice appeared to be largely protected from the fibrotic effects of bleomycin. The lung hydroxyproline content of bleomycin-treated PAI-1 -/- mice was markedly lower than that of bleomycin-treated PAI-1 +/+ mice ($P < 0.001$) and was not significantly different from the level of mice receiving only PBS ($P = 0.63$). The hydroxyproline content observed in PAI-1 +/- mice treated with bleomycin was intermediate, revealing a direct correlation between PAI-1 gene dose and lung hydroxyproline content ($r = 0.72$; $P = 0.001$). No difference in hydroxyproline content of PAI-1 -/- and PAI-1 +/- mice was observed in the absence of bleomycin treatment (data not shown).

Histology. To provide a visual correlate to the quantitative hydroxyproline data, Masson trichrome-stained sections of lung tissue were examined using light microscopy. The lungs of

animals receiving intratracheal PBS appeared normal, regardless of their PAI-1 status. Stained collagen in the lungs of these animals was detectable only in thin bands immediately adjacent to large vessels and airways (data not shown). After 0.075 U bleomycin, the lungs of transgenic mice overexpressing PAI-1 contained dense bands of collagen replacing large areas of lung parenchyma, an example of which is shown in Fig. 3 B. The areas of abnormal stainable collagen in the lungs of normal mice receiving the same dose of bleomycin were fewer in number and considerably less dense (Fig. 3 A). When a higher dose of bleomycin (0.15 U) was administered to normal PAI-1 +/+ mice, a severe fibrotic response was seen. The lungs of these animals contained multiple areas of darkly stained collagen as demonstrated in Fig. 3 C. In marked contrast, the lung tissue of PAI-1 -/- mice receiving 0.15 U of bleomycin appeared essentially normal (Fig. 3 D). Of note, areas of lung tissue with increased collagen also contained increased numbers of inflammatory cells.

Immunohistochemical staining for fibrin. To determine if fibrin accumulation in lung parenchyma correlated with the extent of fibrosis and PAI-1 gene status, lung sections were stained with an antifibrin antibody (Fig. 4). Immunostainable fibrin that was contained in lungs of PAI-1-overexpressing, bleomycin-treated (0.075 U) mice appeared much more abundant than that of bleomycin-treated, normal littermates. The red-staining fibrin was present in both airspaces and interstitium and was located within and adjacent to areas of inflammation and fibrosis. Wild-type mice (PAI-1 +/+) receiving a higher dose of bleomycin (0.150 U) also showed extensive areas of fibrin deposition, while PAI-1 -/- littermates receiving the same dose of bleomycin had no fibrin staining. Mice that received only PBS had no stainable fibrin. Serial sections from each of the tissue blocks had no red-staining when exposed to preimmune serum.

Discussion

The normal alveolar space has net fibrinolytic activity and is proficient at clearing extravascular fibrin by producing u-PA (1, 2). However, during both acute and chronic inflammatory lung diseases, the fibrinolytic process becomes impaired due to increased levels of inhibitors to plasminogen activators (e.g., PAI-1) and plasmin. Plasmin and/or plasminogen activators can participate in many processes including cell migration (25), extracellular matrix remodeling (26–30), generation of inflammatory peptides (31, 32), and activation of latent cytokines and enzymes including metalloproteinases such as collagenases (26, 29). Therefore, we hypothesized that the degree of pulmonary fibrosis that follows inflammatory lung injury would be influenced by the state of the fibrinolytic system. To test this hypothesis, we used transgenic mice with altered PAI-1 gene expression to examine the relationship between the fibrinolytic system and the development of pulmonary fibrosis after inflammatory injury.

PAI-1, a rapid inhibitor of u-PA and t-PA activity, occurs in the lung either as a product of pulmonary alveolar epithelial cells (33, 34) or by transfer from plasma where it is a normal constituent. Transgenic animals that are either deficient in or overexpress PAI-1 have been used to study the functional role of PAI-1. Mice totally deficient in PAI-1 appear normal under basal conditions (18). However, when preformed clots are embolized to their lungs, the clots are lysed more rapidly than

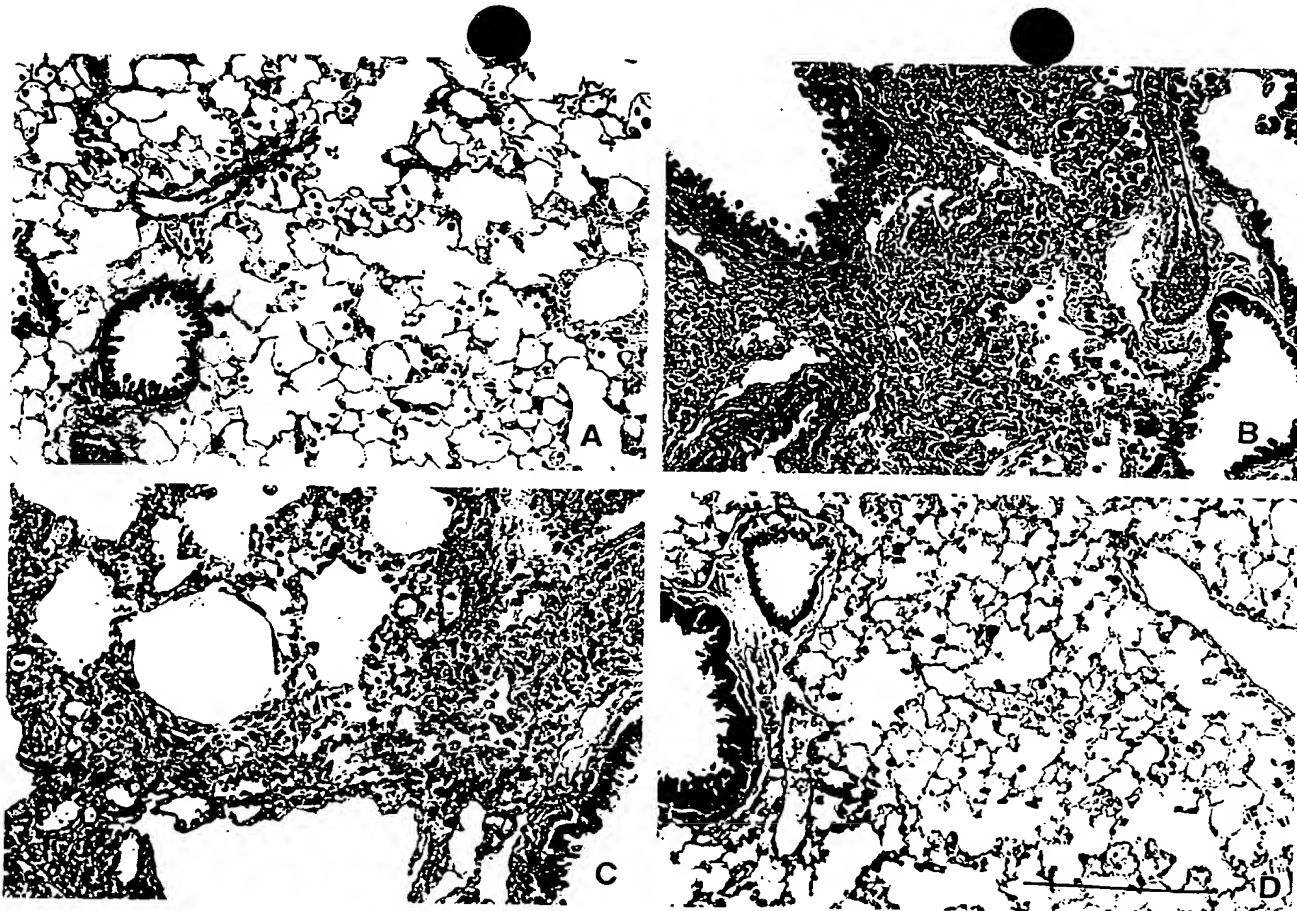


Figure 3. Photomicrographs of Masson trichrome-stained sections of lung tissue from bleomycin-treated mice. (A) Normal mice 2 wk after 0.075 U bleomycin. (B) PAI-1-overexpressing mice 2 wk after 0.075 U bleomycin. (C) Normal mice (PAI-1 +/+) 3 wk after 0.15 U bleomycin. (D) PAI-1 homozygous deficient mice (PAI-1 -/-) 3 wk after receiving 0.15 U bleomycin. The photomicrographs were selected to illustrate the pattern and extent of fibrosis present in the abnormal regions of lung for each experimental group. All panels are at the same magnification; bar, 200 μ m.

they are in control animals. In addition, the PAI-1-deficient animals are less likely to develop venous thrombi after foot pad injection of endotoxin. Conversely, mice expressing high levels of a human PAI-1 transgene develop spontaneous thromboses within their tail and hind leg veins (35). Since these studies showed that fibrin clearance can be influenced by manipulations in PAI-1 gene expression, this approach appeared suitable for investigating the linkage between fibrinolysis and the development of pulmonary fibrosis. The importance of the plasminogen activation system in clearance of fibrin from the lung is further demonstrated by the observation that mice with combined deficiency of t-PA and u-PA, the two major plasminogen activators, exhibit spontaneous fibrin deposition in lung tissue (36).

Bleomycin administration to animals has been widely used to study the pathogenesis of pulmonary fibrosis (14, 23, 37–39). When instilled intratracheally into mice, bleomycin causes a pneumonitis that progresses to fibrosis in a dose-dependent manner with increased collagen content occurring as early as 2 wk after instillation (23). The fibrogenic effects of bleomycin can be attenuated by suppressing immune or inflammatory responses (23, 40, 41). Lung tissue obtained from bleomycin-injured animals has histologic features that are similar to those

seen in the lungs of humans with the adult respiratory distress syndrome and idiopathic pulmonary fibrosis. Of particular relevance to the present study, bleomycin treatment of animals suppresses the fibrinolytic activity of BAL fluid in a pattern similar to that seen in human inflammatory lung diseases (14, 38). Furthermore, intratracheal administration of recombinant human u-PA to rats 30 d after bleomycin exposure can temporarily reduce the amount of lung fibrosis (39).

The results of this study demonstrate a strong relationship between PAI-1 gene dose and the degree of pulmonary fibrosis that follows bleomycin administration. In particular, PAI-1-overexpressing mice experienced greater fibrosis than wild-type mice, while mice homozygous deficient for PAI-1 were protected from fibrosis, with heterozygotes showing an intermediate effect. As mentioned above, there are multiple mechanisms by which alterations in fibrinolytic activity could influence the development of fibrosis. One such mechanism is the ability of plasmin to degrade extravascular fibrin and thus remove the provisional matrix on which fibroblasts invade to form collagen. In support of this, we found that fibrin deposits were localized within and around areas of fibrotic lung. However, other mechanisms might link the fibrinolytic system to scar formation. Since the intensity of bleomycin-induced in-

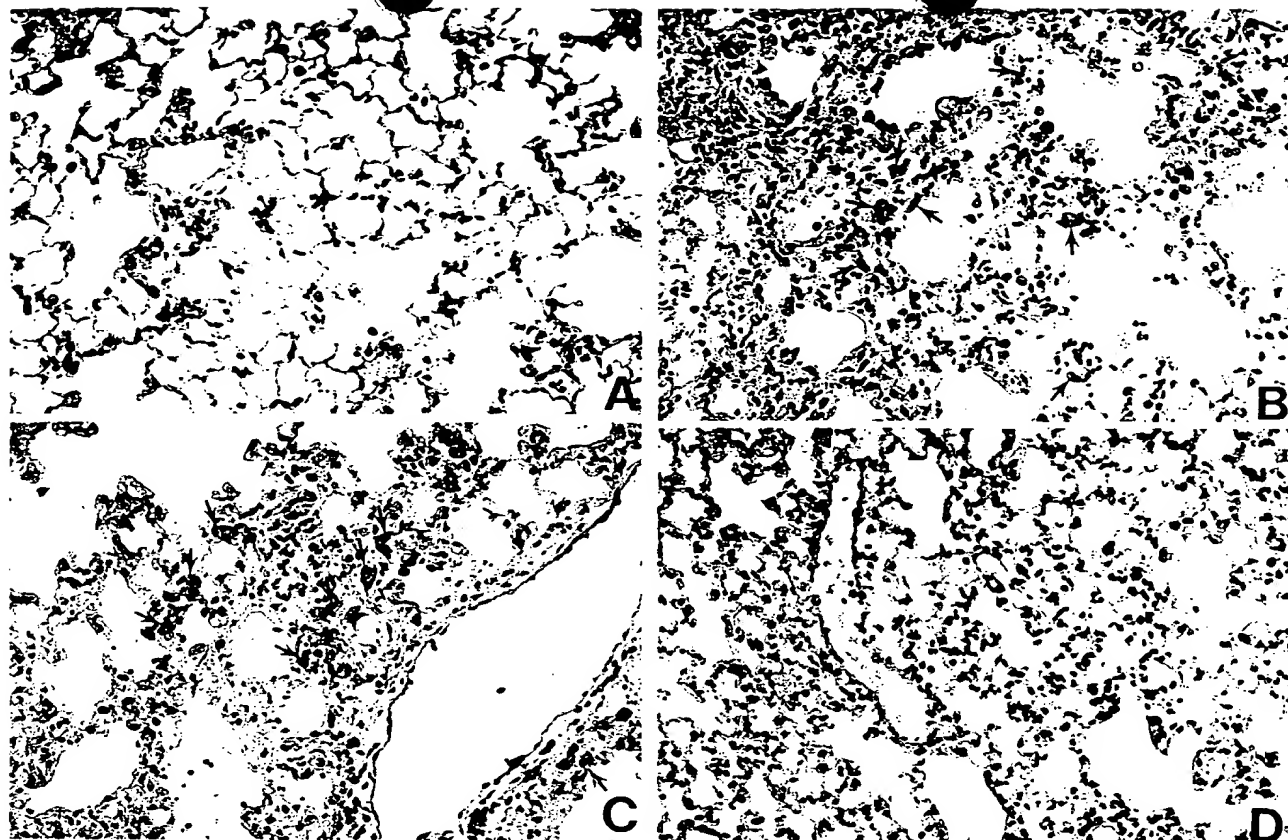


Figure 4. Photomicrographs of immunostained lung tissue from bleomycin-treated mice localizing sites of fibrin deposition. (A) Normal mice 2 wk after 0.075 U bleomycin. (B) PAI-1-overexpressing mice 2 wk after 0.075 U bleomycin. (C) Normal mice (PAI-1 +/+) 3 wk after 0.15 U bleomycin. (D) PAI-1 homozygous deficient mice (PAI-1 -/-) 3 wk after receiving 0.15 U bleomycin. Arrows indicate material staining positively with a goat antiserum against murine fibrin. No antibody binding was seen using control antiserum. The photomicrographs were selected to illustrate the pattern and extent of fibrin deposition in the abnormal regions of lung for each experimental group. All panels are at the same magnification; bar, 200 μ m.

Inflammation is known to correlate with the degree of subsequent fibrosis (42), the fibrinolytic system may exert its effect in part by modulating various inflammatory processes mentioned above. Although plasmin activation has been implicated in the process of inflammatory cell migration, overexpression of PAI-1 does not appear to prevent accumulation of leukocytes in bleomycin-treated mice. Apparently, residual or redundant proteolytic mechanisms are adequate to allow inflammatory cells to infiltrate the lungs.

In summary, we have shown that alterations in the fibrinolytic environment of the alveolar space during inflammatory injury influence the subsequent development of pulmonary fibrosis. These findings suggest that therapeutic interventions designed to enhance fibrinolysis such as administration of either plasminogen activators (39) or inhibitors of PAI-1 (43) may limit the development of pulmonary fibrosis that occurs as a consequence of acute or chronic inflammatory lung diseases.

Acknowledgments

We would like to thank Désiré Collen and Peter Carmeliet for providing the PAI-1-deficient mice, Susan Shollenberger for technical assistance during lung harvests, and Sem H. Phan for advice concerning the bleomycin animal model.

This work was supported by National Institutes of Health grants HL-09104 (to D. T. Eitzman), HL-02728 (to W. P. Fay), HL-39137 (to D. Ginsburg), and HL-46487 (to R. H. Simon), and a Research Training Fellowship from the American Lung Association of Michigan (to R. D. McCoy). D. Ginsburg is a Howard Hughes Medical Institute investigator.

References

- Bertozi, P., B. Astedt, L. Zenzius, K. Lynch, F. LeMaire, W. Zapol, and H. J. Chapman. 1990. Depressed bronchoalveolar urokinase activity in patients with adult respiratory distress syndrome. *N. Engl. J. Med.* 322:890-897.
- Idell, S., K. K. James, E. G. Levin, B. S. Schwartz, N. Manchanda, R. J. Maunder, T. R. Martin, J. McLarty, and D. S. Fair. 1989. Local abnormalities in coagulation and fibrinolytic pathways predispose to alveolar fibrin deposition in the adult respiratory distress syndrome. *J. Clin. Invest.* 84:695-705.
- Bachofen, A., and E. R. Weibel. 1977. Alterations of the gas exchange apparatus in adult respiratory insufficiency associated with septicemia. *Am. Rev. Respir. Dis.* 116:589-615.
- Burkhardt, A. 1989. Alveolitis and collapse in the pathogenesis of pulmonary fibrosis. *Am. Rev. Respir. Dis.* 140:513-524.
- Kuhn, C., J. Boldt, T. J. King, E. Crouch, T. Vartio, and J. A. McDonald. 1989. An immunohistochemical study of architectural remodeling and connective tissue synthesis in pulmonary fibrosis. *Am. Rev. Respir. Dis.* 140:1693-1703.
- Basset, F., V. J. Ferrans, P. Soler, T. Takemura, Y. Fukuda, and R. G. Crystal. 1986. Intraluminal fibrosis in interstitial lung disorders. *Am. J. Pathol.* 122:443-461.
- Fukuda, Y., M. Ishizaki, Y. Masuda, G. Kimura, O. Kawanami, and Y. Masugi. 1987. The role of intraalveolar fibrosis in the process of pulmonary

- structural remodeling in patients with diffuse alveolar damage. *Am. J. Pathol.* 126:171-182.
8. Peyrol, S., J. F. Cordier, and J. A. Grimaud. 1990. Intra-alveolar fibrosis of idiopathic bronchiolitis obliterans-organizing pneumonia. Cell-matrix patterns. *Am. J. Pathol.* 137:155-170.
9. Crouch, E. 1990. Pathobiology of pulmonary fibrosis. *Am. J. Physiol.* 259:L159-L184.
10. Chapman, H. A., C. L. Allen, and O. L. Stone. 1986. Abnormalities in pathways of alveolar fibrin turnover among patients with interstitial lung disease. *Am. Rev. Respir. Dis.* 133:437-443.
11. Hasday, J. L., P. R. Bachwich, J. P. Lynch, and R. G. Sitrin. 1988. Procoagulant and plasminogen activator activities of bronchoalveolar fluid in patients with pulmonary sarcoidosis. *Exp. Lung Res.* 14:261-278.
12. Viscardi, R. M., K. Broderick, C. C. Sun, L. A. Yale, A. Hessamfar, V. Taciak, K. C. Burke, K. B. Koenig, and S. Idell. 1992. Disordered pathways of fibrin turnover in lung lavage of premature infants with respiratory distress syndrome. *Am. Rev. Respir. Dis.* 146:492-499.
13. Idell, S., K. K. James, and J. J. Coalson. 1992. Fibrinolytic activity in bronchoalveolar lavage of baboons with diffuse alveolar damage: trends in two forms of lung injury. *Crit. Care Med.* 20:1431-1440.
14. Idell, S., K. K. James, C. Gillies, D. S. Fair, and R. S. Thrall. 1989. Abnormalities of pathways of fibrin turnover in lung lavage of rats with oleic acid and bleomycin-induced lung injury support alveolar fibrin deposition. *Am. J. Pathol.* 135:387-399.
15. Idell, S., J. Peters, K. James, D. S. Fair, and C. C. Coalson. 1989. Local abnormalities in coagulation and fibrinolytic pathways that promote alveolar fibrin deposition in the lungs of baboons with diffuse alveolar damage. *J. Clin. Invest.* 84:181-193.
16. Loskutoff, D. J., M. Sawdey, and J. Mimuro. 1989. Type I plasminogen activator inhibitor. *Prog. Hemostasis Thromb.* 9:87-115.
17. Fay, W. P., A. D. Shapiro, J. L. Shih, R. R. Schleef, and D. Ginsburg. 1992. Complete deficiency of plasminogen-activator inhibitor type 1 due to a frame-shift mutation. *N. Engl. J. Med.* 327:1729-1733.
18. Carmeliet, P., J. M. Stassen, L. Schoonjans, B. Ream, J. J. van den Oord, M. De Mol, R. C. Mulligan, and D. Collen. 1993. Plasminogen activator inhibitor-1 gene-deficient mice. II. Effects on hemostasis, thrombosis, and thrombolysis. *J. Clin. Invest.* 92:2756-2760.
19. Hamsten, A., B. Wiman, U. de Faire, and M. Blomback. 1985. Increased plasma levels of a rapid inhibitor of tissue plasminogen activator in young survivors of myocardial infarction. *N. Engl. J. Med.* 313:1557-1563.
20. Wiman, B., and A. Hamsten. 1990. The fibrinolytic enzyme system and its role in the etiology of thromboembolic disease. *Semin. Thromb. Hemostasis.* 16:207-216.
21. Carmeliet, P., L. Kieckens, L. Schoonjans, B. Ream, A. van Nuffelen, G. Prendergast, M. Cole, R. Bronson, D. Collen, and R. C. Mulligan. 1993. Plasminogen activator inhibitor-1 gene-deficient mice. I. Generation by homologous recombination and characterization. *J. Clin. Invest.* 92:2746-2755.
22. Miller, S. A., D. D. Dykes, and H. F. Polesky. 1988. A simple salting out procedure for extracting DNA for human nucleated cells. *Nucleic Acids Res.* 16:1215.
23. Schrier, D. J., S. H. Phan, and B. M. McGarry. 1983. The effects of the nude (nu/nu) mutation on bleomycin-induced pulmonary fibrosis. A biochemical evaluation. *Am. Rev. Respir. Dis.* 127:614-617.
24. Woessner, J. F. 1961. The determination of hydroxyproline in tissue and protein samples containing small proportions of this amino acid. *Arch. Biochem. Biophys.* 93:440-447.
25. Kirchheimer, J. C., and H. G. Remold. 1989. Endogenous receptor-bound urokinase mediates tissue invasion of human monocytes. *J. Immunol.* 143:2634-2639.
26. Werb, Z., C. L. Mainardi, C. A. Vater, and E. D. Harris. 1977. Endogenous activation of latent collagenase by synovial cells: evidence for a role for plasminogen activator. *N. Engl. J. Med.* 296:1017-1021.
27. Liotta, L. A., R. H. Goldfarb, R. Brundage, G. P. Siegal, V. Terranova, and S. Garbisa. 1981. Effect of plasminogen activator (urokinase), plasmin, and thrombin on glycoprotein and collagenous components of basement membrane. *Cancer Res.* 41:4629-4636.
28. Mochan, E., and T. Keler. 1984. Plasmin degradation of cartilage proteoglycan. *Biochim. Biophys. Acta.* 800:312-315.
29. Chapman, H. A., and O. J. Stone. 1984. Cooperation between plasmin and elastase in elastin degradation by intact murine macrophages. *Biochem. J.* 222:721-728.
30. Laiho, M., and J. Keski-Oja. 1989. Growth factors in the regulation of pericellular proteolysis: a review. *Cancer Res.* 49:2533-2553.
31. Dang, C. V., W. R. Bell, D. Kaiser, and A. Wang. 1985. Disorganization of cultured vascular epithelial cell monolayers by fibrinogen fragment D. *Science (Wash. DC).* 227:1487-1490.
32. Senior, R. M., W. F. Skugen, G. L. Griffin, and G. D. Wilner. 1986. Effects of fibrinogen derivatives upon the inflammatory response. Studies with human fibrinopeptide B. *J. Clin. Invest.* 77:1014-1019.
33. Gross, T. J., R. H. Simon, C. J. Kelly, and R. G. Sitrin. 1991. Rat alveolar epithelial cells concomitantly express plasminogen activator inhibitor-1 and urokinase. *Am. J. Physiol.* 260:L286-L295.
34. Marshall, B., B. Brown, M. Rothstein, N. Rao, J. Hoidal, and G. Rodgers. 1991. Alveolar epithelial cells express both plasminogen activator and tissue factor. *Chest.* 99(Suppl. 3):25S-27S.
35. Erickson, L. A., G. J. Fici, J. E. Lund, T. P. Boyle, H. G. Polites, and K. R. Marotti. 1990. Development of venous occlusions in mice transgenic for the plasminogen activator inhibitor-1 gene. *Nature (Lond.).* 346:74-76.
36. Carmeliet, P., L. Schoonjans, L. Kieckens, B. Ream, J. Degen, R. Bronson, R. De Vos, J. J. van den Oord, D. Collen, and R. C. Mulligan. 1994. Physiological consequences of loss of plasminogen activator gene function in mice. *Nature (Lond.).* 368:419-424.
37. Adamson, I. Y., and D. H. Bowden. 1974. The pathogenesis of bleomycin-induced pulmonary fibrosis. *Am. J. Pathol.* 77:185-197.
38. Idell, S., K. K. Gonzales, C. K. MacArthur, C. Gillies, P. N. Walsh, J. McLarty, and R. S. Thrall. 1987. Bronchoalveolar lavage procoagulant activity in bleomycin-induced lung injury in marmosets. Characterization and relationship to fibrin deposition and fibrosis. *Am. Rev. Respir. Dis.* 136:124-133.
39. Hart, D. A., P. Whidden, F. Green, J. Henkin, and D. E. Woods. 1994. Partial reversal of established bleomycin-induced pulmonary fibrosis by rhurokinase in a rat model. *Clin. Invest. Med.* 17:69-76.
40. Thrall, R. S., S. H. Phan, J. R. McCormick, and P. A. Ward. 1981. The development of bleomycin-induced pulmonary fibrosis in neutrophil-depleted and complement-depleted rats. *Am. J. Pathol.* 105:76-81.
41. Phan, S. J., R. S. Thrall, and C. Williams. 1981. Bleomycin-induced pulmonary fibrosis. Effects of steroid on lung collagen metabolism. *Am. Rev. Respir. Dis.* 124:428-434.
42. Shen, A. S., C. Haslett, D. C. Feldsien, P. M. Henson, and R. M. Charniak. 1988. The intensity of chronic lung inflammation and fibrosis after bleomycin is directly related to the severity of acute injury. *Am. Rev. Respir. Dis.* 137:564-571.
43. Eitzman, D. T., W. P. Fay, D. A. Lawrence, A. Francis-Chmura, J. D. Shore, S. T. Olson, and D. Ginsburg. 1995. Peptide-mediated inactivation of recombinant and platelet plasminogen activator inhibitor-1 in vitro. *J. Clin. Invest.* 95:2416-2420.